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Engineering *Lactococcus lactis* for the production of unusual anthocyanins using tea as substrate

Ana Solopova[a,1], Amanda Y. van Tilburg[a], Alexandre Foito[b], J. William Allwood[b], Derek Stewart[b,c], Saulius Kulakauskas[d], Oscar P. Kuipers[a,*]

[a] Molecular Genetics, University of Groningen, Groningen, Netherlands
[b] Environmental and Biochemical Sciences Group, The James Hutton Institute, Dundee, UK
[c] School of Engineering and Physical Sciences, Institute of Mechanical, Process and Energy Engineering, Heriot-Watt University, Edinburgh, UK
[d] Micalis Institute, INRA, AgroParisTech, Université Paris-Saclay, 78350 Jouy-en-Josas, France

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ABSTRACT

Plant material rich in anthocyanins has been historically used in traditional medicines, but only recently have the specific pharmacological properties of these compounds been the target of extensive studies. In addition to their potential to modulate the development of various diseases, coloured anthocyanins are valuable natural alternatives commonly used to replace synthetic colourants in food industry. Exploitation of microbial hosts as cell factories is an attractive alternative to extraction of anthocyanins and other flavonoids from plant sources or chemical synthesis. In this study, we present the lactic acid bacterium *Lactococcus lactis* as an ideal host for the production of high-value plant-derived bioactive anthocyanins using green tea as substrate. Besides the anticipated red-purple compounds cyanidin and delphinidin, orange and yellow pyranoanthocyanidins with unexpected methylation patterns were produced from green tea by engineered *L. lactis* strains. The pyranoanthocyanins are currently attracting significant interest as one of the most important classes of anthocyanin derivatives and are mainly formed during the aging of wine, contributing to both colour and sensory experience.

1. Introduction

Various phytochemicals, such as flavonoids are increasingly valued for their health promoting activities (Rodriguez-Mateos et al., 2014; Atanasov et al., 2015; Perez-Vizcaino and Fraga, 2018; Foito et al., 2018). They are simple compounds present in fresh fruits and vegetables, or complex compounds present in bark, roots and leaves of plants. Flavonoids have become a topic of increasing interest not only because of their antioxidant properties but also due to their beneficial effects on health. Whilst they can act as free radical scavengers, metal chelators and singlet oxygen quenchers reducing lipid peroxidation, DNA damage and stimulating the expression of detoxification enzymes, these compounds demonstrate promising effects in the combat of cardiovascular disease, certain types of cancer, neurodegenerative diseases, diabetes and inflammation (Foito et al., 2018).

A group of flavonoids, known as anthocyanins, is responsible for the colour of many fruits, vegetables and flowers. Anthocyanins encompass a large group of compounds, with over 600 known molecular structures. However, this diversity is based on six naturally occurring anthocyanidins: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. They differ from each other by the hydroxylation and/or methoxylation pattern of ring B, which affects directly the hue and colour stability (de Freitas et al., 2017). Research activity into anthocyanins has increased in recent years, mainly driven by interest in their bioactive properties and colour properties (Khoo et al., 2017). Recently, there have been increasing efforts in reducing the use of synthetic colourants in the food industry and thus anthocyanins are increasingly being used as natural, healthier colour alternatives. Anthocyanins and other flavonoids are mainly extracted from plants and as result are subject to seasonality of raw material, variation of abundance in different species, and fluctuations in the abundance of these compounds driven by environmental variables. Additionally, the purification of a single chemical from complex plant matrices is often difficult due to the presence of structurally similar compounds. Plant cell cultures provide

* Corresponding author.
E-mail addresses: ana.solopovabuivyde@ucc.ie (A. Solopova), a.y.van.tilburg@rug.nl (A.Y. van Tilburg), Alex.Foito@hutton.ac.uk (A. Foito), will.allwood@hutton.ac.uk (J.W. Allwood), derek.stewart@hutton.ac.uk (D. Stewart), saulius.kulakauskas@inra.fr (S. Kulakauskas), o.p.kuipers@rug.nl (O.P. Kuipers).
[1] Present address: APC Microbiome Institute, University College Cork, Cork, Ireland.

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a promising strategy for the production of specific molecules, but to date they have had limited commercial success in food biotechnology applications as result of limited culture yields and/or poorly optimized production systems (Davies and Deroles, 2014; Appelhagen et al., 2018). Chemical synthesis of anthocyanins is complex and often produces large amounts of toxic waste. The exploitation of microbial hosts as cell factories for the production of various phytochemicals is an attractive environmentally-friendly and increasingly cost-effective alternative. The fast growth of bacteria allows short production times and generally chemically distinct structure of the product facilitates easy purification (Marienhagen and Bott, 2013; Lim et al., 2015; Stahlhut et al., 2015; Dudnik et al., 2017). Many plant pathways have been successfully reconstructed and expressed in microorganisms so far. However, almost all of them employed *Escherichia coli* and *Saccharomyces cerevisiae* as production hosts (Marienhagen and Bott, 2013; Milke et al., 2018). Lactic acid bacteria, and in particular *L. lactis,* provide an attractive alternative for production of plant high value chemicals. It has a long history of safe usage in food fermentations and has been granted a Generally Regarded As Safe (GRAS) status. Anthocyanins are a great target for heterologous production since their biosynthesis pathways are characterised and accumulation is quickly disclosed by the colour change of the production culture.

Anthocyanins are synthesized from flavanones in the plant cytosol, on the endoplasmic reticulum, and then transported into the vacuole. Anthocyanidin synthase (ANS) and UDP-glucose: anthocyanidin 3-*O*-glucosyltransferase (3GT) are the last two enzymes in the pathway responsible for the formation of a stable colourful product (Ferreyra et al., 2012). ANS belongs to the 2-oxoglutarate iron-dependent oxygenases and was cloned first from perilla (*Perilla frutescens*) (Saito et al., 1999). It uses ferrous iron as a cofactor and 2-oxoglutarate (2OG) as a co-substrate (Saito et al., 1999; Wilmouth et al., 2002). The enzyme requires an unusually high concentration of ascorbate for optimal turnover. ANS was postulated to catalyse the reaction from the colourless leucoanthocyanidins to the coloured anthocyanidins (Saito et al., 1999). Later *in vitro* activity studies revealed that the selectivities of 2OG-dependent oxygenases that are involved in flavonoid synthesis overlap. ANS was shown to have properties of a flavanol synthase FLS and catalyse oxidation of dihydroquercetin to quercetin (Wilmouth et al., 2002). ANS from *Gerbera hybrida* and *Petunia hybrida* accepted (+)-catechin as a substrate to form cyanidin, quercetin and an oxidized (+)-catechin dimer (Wellmann et al., 2006; Yan et al., 2008).

In this study, we engineered the food-grade bacterium *L. lactis* for anthocyanin production using flavan-3-ols as substrates. In order to demonstrate the feasibility of utilizing feedstocks naturally rich in flavan-3-ols we successfully converted green tea infusion into a variety of unusual and valuable pigments. We showed that the polysaccharides of the thick cell wall of the Gram-positive bacterium might retain polyphenols and investigated multiple options to overcome this barrier. Besides the classical anthocyanins, the engineered *L. lactis* strains were able to produce various red, orange and yellow cyanidin and delphinidin derivatives reaching total milligram-per-litre production titres. Some of the compounds belong to an intriguing class of methylpyranoanthocyanins that are known to form during ageing of wine. The approach used here offers a new environmentally friendly strategy for obtaining anthocyanin-rich fermentation products from various polyphenol-rich waste streams.

2. Materials and methods

2.1. Bacterial strains, plasmids and culturing conditions

All strains and vectors used in this study are listed in Table 1. *L. lactis* was grown as standing cultures at 30 °C in M17 broth (Difco, Sparks, MD) containing 0.5% (wt/vol) glucose or on solid GM17 with 1.5% (wt/vol) agar. *E. coli* DH5α was used as the host for cloning and grown in LB medium at 37 °C with shaking or on LB medium solidified with 1.5% (wt/vol) agar. When required, the growth media were supplemented with the following antibiotics: 150 μg/ml erythromycin (Em) for *E. coli*, 1.5 μg/ml erythromycin or 5 μg/ml chloramphenicol (Cm) for *L. lactis*.

2.2. General DNA manipulation techniques

DNA manipulations were done essentially as described (Sambrook, 1989). Plasmid DNA and PCR products were isolated and purified using the Nucleospin Plasmid kit and NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel, Düren, Germany), respectively, according to the manufacturer's instructions. Restriction enzymes, T4 DNA ligase, Dream Taq DNA-polymerase, Phusion DNA Polymerase were obtained from Thermofisher Scientific (Waltham, MA, USA) and used according to the supplier's guidelines.

2.3. Construction of anthocyanin producer strains

3GT- and ANS-encoding genes from *A. thaliana* were optimized for expression in *L. lactis* using Optimizer (Puigbò et al., 2007) and synthesized by GeneScript (Piscataway, NJ, US), (See Table 2). Four codon optimized variants of *A. thaliana* ANS (AT4G22880, NCBI: NP_194019.1) were synthesized by ATUM (Newark, CA, US). *Ipomea nil* (NCBI: XP_019172730.1) and *Gerbera hybrida var. tacora ANS* (GenBank: AAY15743.2) genes optimized for expression in yeast *Saccharomyces cerevisiae* were received as kind gift from Evolva (Basel, Switzerland). In order to evaluate expression, ANS-encoding genes from various sources were first cloned into pNZ8048 via NcoI-XbaI restriction sites with a Strep-tag extension (for details see the Western blot section below). ANS A/B/C/D-encoding genes were PCR-amplified and ligated into pNZ8048 (de Ruyter et al., 1996) digested with restriction enzymes NcoI and HindIII (Supplementary Table 1). Vectors obtained were introduced in *L. lactis* NZLdh$^-$ and NZLdh$^-$3gt via electroporation (Holo and Nes, 1995).

Native *A. thaliana* 3GT-encoding gene (AT5G1705, GenBank: KJ138682.1) was subcloned from pCDF-At3GT-PhANS which was a kind gift from M. Koffas (Yan et al., 2008). A codon-optimized 3gt was synthesized by GeneScript. To integrate it into *L. lactis* chromosome, a pCS1966-based vector was used (Solem et al., 2008). The 3gt gene was amplified by PCR with primer pair 3GTXhoIFw/3GTBamHIRev using pUC57-3gt from GeneScript as the template. The amplicon was inserted in *L. lactis* integration vector pSEUDO::Pusp45-sfgfp(Bs) as an XhoI/BamHI restriction fragment replacing the resident *gfp* gene. This yielded pSEUDO::Pusp45-*3gt*. The vector was obtained and amplified in *E. coli* DH5α. *L. lactis* strain NZLdh$^-$ pSEUDO::Pusp45-*3gt* was obtained by a double crossover integration of pSEUDO::Pusp45-*3gt* into the *pseudo10* locus on the chromosome of NZLdh$^-$. Integration was performed as described by Defoor et al. and Solem et al. (Defoor et al., 2007; Solem et al., 2008). The chromosomal structure of all obtained strains was confirmed by PCR analysis and sequencing of the modified regions (Macrogen Sequencing, Amsterdam, the Netherlands).

2.4. Western blot

Plant enzymes-encoding genes were amplified using PCR primers extended with a Strep-Tag (coding for W-S-H-P-Q-F-E-K) adding it to the C- or N-terminal part of the protein and cloned into pNZ8048 via NcoI-XbaI restriction sites. The resulting plasmids were used for the transformation of *L. lactis*. In order to check the expression of the probed protein, recombinant strains were cultivated and induced the same way as described for *in vitro* enzymatic activity tests (see below). Cell lysates were prepared by bead-beating the cells in the loading buffer and boiling for 5 min. Tricine- SDS-polyacrylamide gel electrophoresis was performed as described before (Schägger, 2006). After separation by SDS-PAGE, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane by Western blotting. Strep-tag-labelled

Table 1
Bacterial strains and plasmids used in this study.

Strain/plasmid	Description	Reference
E. coli strains		
DH5α	*fhuA2 lac(del)U169 phoA glnV44 Φ80′ lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Laboratory stock
DH5α pSEUDO::Pusp45-*3gt*	DH5α carrying pSEUDO::Pusp45-*3gt*	This study
E. coli plasmids		
pSEUDO::Pusp45-sfgfp(Bs)	pSEUDO derivative carrying constitutive promoter P*usp45* and *gfp* for integration in *L. lactis* chromosome, *pseudo10* locus, Ery^r	Overkamp et al. (2013)
pSEUDO::Pusp45-*3gt*	carries 3gt from *A. thaliana* under control of lactococcal P*usp45* for integration in *L. lactis* chromosome, Ery^r	This study
L. lactis strains		
NZ9000	MG1363 derivative with chromosomally integrated *nisRK* for nisin induction	Kuipers et al. (1998)
NZLdh$^-$	NZ9000 Δ*ldh*Δ*ldhB* – lactate dehydrogenase-negative derivative	Gaspar et al. (2007)
NZLdh$^-$3gt	NZLdh$^-$ with chromosomally integrated P*usp45-3gt* in *pseudo10* locus	This study
NZAnt	NZLdh$^-$3gt carrying pANS_D	This study
NZpANS	NZLdh$^-$ carrying pANS_D	This study
P1	NZLdh$^-$ pANS with IS*981* in *llmg_0026*; pellicle mutant	This study
L. lactis plasmids		
pNZ8048	High copy number vector, carries P*nisA* for nisin induction, Cm^r	Kuipers et al. (1998)
pNZIn	pNZ8048 P*nisA*-ANS from *Ipomea nil*	This study
pNZGh	pNZ8048 P*nisA*-ANS from *Gerbera hybrida var. tacora*	This study
pNZAt	pNZ8048 P*nisA*-ANS from *Arabidopsis thaliana*	This study
pANS_A	pNZ8048 P*nisA*-ANS_A from *A. thaliana*	This study
pANS_B	pNZ8048 P*nisA*-ANS_B from *A. thaliana*	This study
pANS_C	pNZ8048 P*nisA*-ANS_C from *A. thaliana*	This study
pANS_D	pNZ8048 P*nisA*-ANS_D from *A. thaliana*	This study

Table 2
Plant genes assayed for the expression and activity in *L. lactis*. ANS from *A. thaliana* was subjected to two ways of codon optimization to improve its performance in *L. lactis*. Expression level was ranked by the band size and intensity on the Western blot membrane; enzyme activity was ranked based on the colour intensity of the activity assay mixture (see Section 2.4). ANS_D and the codon-optimized 3GT from *A. thaliana* were selected for further strain construction.

Enzyme	Species	Codon-optimized for (Optimization method)	Expression (Western blot)	Activity (*In vitro* assay)	Source
ANS	*Ipomoea nil*	yeast	Not expressed	–	Evolva
ANS	*Gerbera hybrida var. tacora*	yeast	Poor	Moderate	Evolva
ANS	*Arabidopsis thaliana*	*L. lactis* (Optimizer)	Very good	Very good	This study
ANS_A	*A. thaliana*	*L. lactis* (ATUM)	Not tested	Very good	This study
ANS_B	*A. thaliana*	*L. lactis* (ATUM)	Not tested	Poor	This study
ANS_C	*A. thaliana*	*L. lactis* (ATUM)	Not tested	Good	This study
ANS_D	*A. thaliana*	*L. lactis* (ATUM)	Not tested	Very good	This study
3GT	*A. thaliana*	Native	Poor	Not tested	Yan et al. (2008)
3GT	*A. thaliana*	*L. lactis*	Very good	Good	This study

proteins were detected using streptactin-horseradish peroxidase conjugate (Iba solutions, Göttingen, Germany) and ECL Prime detection kit (GE Healthcare, Waukesha, WI, US) according to the manufacturer's instructions.

2.5. In vitro enzyme activity assays

L. lactis strains expressing heterologous genes were grown in 5 ml of GM17 medium with 5 μg/ml Cm if ANS was assayed, or with 1.5 μg/ml Ery if 3GT was assayed. The following day the preculture was transferred to 50 ml of GM17 supplemented with appropriate antibiotics. For ANS induction, 1 ng/ml nisin was added when the culture reached an OD600 of 0.2. After 4 h of growth cells were collected by centrifugation. Cell pellet was resuspended in an appropriate cold reaction buffer (4 °C) and disrupted by bead-beating. The resulting crude extract was used in the enzymatic assay. ANS activity assay was adapted from a protocol published earlier (Yan et al., 2008). The reaction mixture consisted of 0.1 mM (+)-catechin, 2.5 mM ascorbic acid, 1 mM 2-oxoglutarate, 0.4 mM $FeSO_4$, 2 mg/ml bovine catalase and 60 μl crude extract in 200 mM potassium phosphate buffer pH 6.0. Reactions of 100 μl were incubated at 30 °C for 2 h. The reaction was stopped and the product stabilized with 2 μl 37% HCl. 100% MeOH was added and HPLC analysis was performed to confirm the presence of cyanidin. Red colour intensity directly indicated a successful reaction and correlated well with HPLC data. Enzymes that were catalysing reactions yielding the most intense colour were selected for further strain construction.

3GT activity assay reaction mixture (100 μl) consisted of 0.2 mM pelargonidin, 1 mM UDP-glucose and crude extract in 100 mM Tris pH 6.5, 5 mM $MgCl_2$, 1 mM KCl buffer pH 6.5. The mixture was incubated at 30 °C for 2 h. The reaction was stopped by addition of 2N HCl, diluted with methanol and analysed by HPLC for the presence of pelargonidin 3-*O*-Glu.

2.6. Isolation of pellicle mutants and mapping of mutations in a polysaccharide synthesis gene cluster in their chromosomes

To isolate pellicle mutants from NZLdh$^-$, we adapted earlier described procedure (Chapot-Chartier et al., 2010). Briefly, we first isolated mutant derivatives, resistant to the lactococcal virulent small isometric-headed phage sk1, specific to MG1363 (Chandry et al., 1997). The mutants were then tested microscopically for long chain formation, the phenotype of pellicle-negative mutation. Further, the chromosomal 3175 bp fragment containing *llmg_0226* gene of each phage-resistant isolate was PCR-amplified with Phusion DNA Polymerase using primers 229 and 226 (Supplementary Table 1; Chapot-Chartier et al., 2010). An increase in the PCR fragment size (approx. 5000 bp instead of expected 3175 bp) suggested the presence of an IS element. The DNA nucleotide sequence of the amplified fragments was determined by Macrogen Sequencing (Amsterdam, the Netherlands). Comparison of the obtained DNA sequence with that of the *L. lactis* NZ9000 genome allowed the identification of an IS*981* integration in the *llmg_0226* gene. The mutant, carrying IS*981* insertion in *llmg_0226* gene was transformed with

plasmid pANS, named as P1 and used for further studies.

2.7. Preparation of tea infusions enriched in polyphenols

Two commercial green tea infusions – Lipton Green Tea Classic and Tetley Green Tea – were prepared by adding 200 ml of hot water (≈90 °C) to one tea bag. After 4 min the tea bag was removed and the solution was cooled down. The total phenolic content was determined using Folin-Ciocalteau method, adapted to a microplate reader (Tavares et al., 2010). Tea infusion was loaded on Solid phase extraction (SPE) column STRATA C18; after the washing step, 20% acetonitrile and 0.5% of acetic acid in water was used for elution. The first 2 ml were discarded and only when the coloured fraction reached the end of the column, the volume was collected. Methanol with 2% of formic acid was added and the flow-through was collected. Finally, 0.5% acetic acid in acetonitrile was added and the eluent collected. The total phenolic content was calculated as mM gallic acid equivalent measurement by using gallic acid calibration curve. The tea fractions were freeze-dried and stored at −20 °C.

2.8. Anthocyanin biosynthesis and extraction

Unless stated otherwise, anthocyanin production was carried out as follows: *L. lactis* was grown in 5 ml of GM17 medium supplemented with 5 μg/ml Cm overnight. The following day 3 ml of the preculture was transferred to 100 ml of GM17. When the culture reached an OD_{600} of 0.2, 1 ng/ml nisin was added to induce P*nisA-ans*. After 5 h cells were collected by centrifugation. The resulting cell pellet was resuspended in 20 ml of chemically-defined medium (CDM) (Goel et al., 2012) containing 1% glucose, 10 mM 2-oxoglutarate, 2.5 mM ascorbic acid and a substrate: 1.5 mM (+)-catechin or tea infusion containing approximately 4–14 mM of (+)-catechin as estimated by gallic acid content. This medium is referred to as "production medium" throughout the text. To facilitate diffusion of the substrate through the thick cell wall of *L. lactis* 1 mg/ml of lysozyme and 2.25 ng/ml of nisin was added to the production medium. Cultures with all the components were then incubated at 30 °C with slow shaking (140–170 rpm) for 16 h. At the end of incubation pH was adjusted to pH 3 by addition of HCl to stabilize the product. The cells were then collected by centrifugation, and the pellets were stored at −80 °C. To extract polyphenols, 3–4 ml of methanol acidified with trifluoroacetic acid (TFA) to a final concentration of 0.1%, was added to the cell pellets and bead-beating or sonication was applied. The extraction step was repeated until the pellet had lost most of its red colour. The obtained extracts were directly analysed by spectrophotometer (Beckman Coulter DU-730, CA, USA) at 515 nm or using HPLC, or freeze-dried and stored at −20 °C for further analysis by LC-MS or ^{1}H-NMR.

2.9. Anthocyanin analysis by HPLC

The methanol extracts were analysed using Agilent 1260 Infinity II HPLC with an analytical Aeris Widepore 3.6 μm XB-C18 250 × 4.60 mm column. A flow rate of 0.85 ml/min was used with a linear grade of 0.1% TFA in water (mobile phase A) and acetonitrile (mobile phase B) by the following method: 0–2 min 5% B, 2–12 min 5–40% B, 13–19 min 40–60% B, 19–22 min 60 to 5% B. Under these conditions, cyanidin 3-*O*-glucoside was detected at 17 min, cyanidin at 19.5 min, pyranoanthocyanidins 3, 4, 5 and 6 were detected at 20, 20.7, 21.3 and 22 min, respectively. Authentic cyanidin 3-*O*-glucoside, cyanidin, pelargonidin and (+)-catechin (Sigma-Aldrich, Darmstadt, Germany) were used as standards.

2.10. Anthocyanin analysis by LC-MS

Freeze-dried extracts from production cultures were reconstituted in 1 ml of 0.1% formic acid acetonitrile (ACN) aqueous solution (50:50). From these extracts, 450 μl were filtered in 0.45 μm filter vials and subsequently analysed by LC-MS in both positive and negative modes. Samples were then analysed by LC-MS on an HPLC consisting of an Accela 600 quaternary pump and an Acella PDA detector coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK) with accurate mass capabilities, operated under the Xcalibur software package 2.0 as described previously (McDougall et al., 2014). Five microliters of the samples were injected in a Synergi Hydro RP-80 Å 150 × 2mm (Phenomenex, Torrance, CA, USA) and autosampler and column temperatures were maintained at 4 °C and 30 °C, respectively. Samples were eluted at a flow rate of 0.3 ml/min using two mobile phases (A: 0.1% formic acid in dH_2O; B: 0.1% formic acid in acetonitrile) as described in Supplementary Table 2. The PDAD scanned discrete channels at 280 nm, 365 nm and 520 nm and continually scanned across 200–700 nm for spectral properties. Mass detection was carried out in both positive and negative ESI modes. Data acquisition was performed in ESI full scan mode within the FT detector operating with a mass resolution of 30,000 (FWHM defined at *m/z* 400), the HESI probe temperature was set to 100 °C, the capillary temperature to 275 °C, the sheath gas at 60 psi and auxiliary gas at 30 psi. For the positive polarity, the source voltage was set at +4.5 kV, capillary voltage at 44 V and tube lens voltage at 100 V. For negative polarity, the source voltage was set at −3.5 kV, capillary voltage at −44 V and tube lens voltage at −100 V. In addition the analysis was repeated in full-scan mode within the FT detector and followed by a data dependent MS/MS of the three most intense ions within the LTQ ion trap detector, using Helium as a collision gas, with a normalized collision energy of 45, mass isolation width of ± 1 *m/z*, Activation Q of 0.25 and activation time of 30 ms, source voltage (set at 3.4 kV) in wide band activation mode. Full scan FT data are aquired in profile mode, whereas the DDA MS2 LTQ fragmentation spectra are aquired in centroid mode. A scan speed of 0.1 s and 0.4 s are applied in the LTQ and FT-MS respectively. The Automatic Gain Control was set to 1×10^5 and 5×10^5 for the LTQ and FT-MS respectively. Peaks were tentatively annotated by utilizing the accurate mass (< 3 ppm), MS/MS fragmentation patterns and reference to retention time for previously analysed standards when available (Supplementary Table 3).

2.11. ^{1}H-NMR analysis

Samples were prepared by dissolving freeze-dried material in methanol-d4/trifluoroacetic acid-d1 9:1 (v/v). ^{1}H-NMR spectra were recorded with an Agilent Technologies 400/54 Premium Shield spectrometer at ambient temperature.

3. Results

3.1. Selection of heterologous genes and functional integration of the pathway in L. lactis

The anthocyanin, cyanidin-3-*O*-glucoside, can be synthesized in two enzymic steps from (+)-catechin (Fig. 1; Wellmann et al., 2006) by the action of ANS and 3GT. To construct a functional pathway for anthocyanin biosynthesis, a library of genes coding for ANS and 3GT from different plant sources was cloned into *L. lactis*. Besides "native" plant genes, codon-optimized variants were tested for their expression (Table 2). The expression of ANS and 3GT were probed by tagging each protein with an N- or C- terminal Strep-tag and using Western blotting to confirm detection of the protein band of the correct size. Successfully synthesized proteins were then tested for their functionality using *in vitro* activity assays for ANS and 3GT (Table 2) and either the nisin inducible promoter or the *usp45* promoter was used to drive expression.

The best expressed genes coding for both, ANS and 3GT originated from *A. thaliana* (Table 2). Although the native 3GT encoding gene was expressed in *L. lactis* (notably, the GC content of genes in both organisms is similar – around 36%), the codon-optimized version of the same

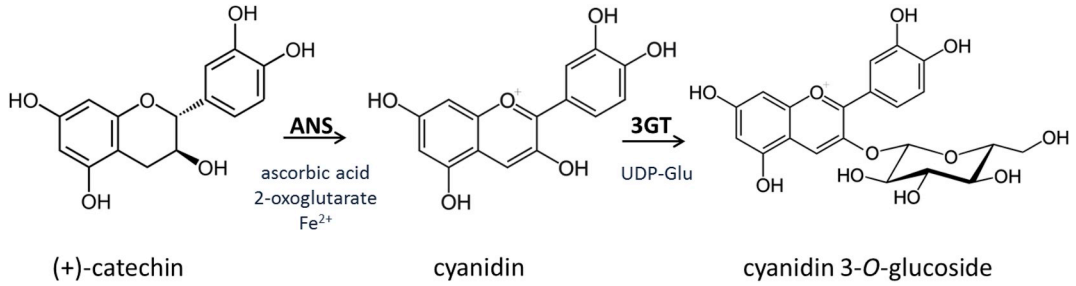

Fig. 1. Required biosynthetic steps for cyanidin 3-*O*-glucoside production in *L. lactis* NZAnt.

gene was translated more efficiently. 3GT and ANS were codon-optimized using Optimizer (Puigbò et al., 2007). This strategy relies on codon adaptation index calculation (CAI) which describes the similarity between the codon usage of the gene of interest and the codon usage of highly expressed *L. lactis* genes such as ribosomal proteins. To obtain the best performing ANS, the gene from *A. thaliana* was subjected to an alternative strategy of optimization which is used by ATUM (Newark, CA, US). To learn more about *L. lactis* codon preference, we tested the expression of 48 green fluorescent proteins that only differed in their codon usage. The expression data obtained from the 48 tested protein variants was used to design an algorithm for codon optimization for *L. lactis*. Based on this information, four ANS variants were synthesized at ATUM. From all the codon-optimized ANS genes, ANS_D was chosen as the highly expressed variant (Table 2).

Thus, the genes coding for ANS and 3GT were cloned together in *L. lactis* in order to obtain an anthocyanin producer. *L. lactis* has a relatively simple metabolism with lactate being the major end-product of the glycolytic pathway. It was previously shown that metabolic engineering of *L. lactis* for production of various compounds often requires disruption of lactate dehydrogenase genes in order to steer the metabolic flux away from lactate production. Instead of the main lactococcal metabolic end-product lactate, a lactate dehydrogenase-negative strain produces a variety of metabolites which allows it to reach higher biomass yield. Furthermore, the strain is expected to have higher levels of acetyl-CoA which is an important precursor of polyphenolic compounds. The strains obtained could potentially be improved for the construction of the full anthocyanin synthesis pathway from amino acids. Indeed, *L. lactis* NZ9000Δ*ldh*Δ*ldhB* (NZLdh$^-$) (Gaspar et al., 2007) was shown to be an excellent host for the production of stilbene resveratrol (Gaspar et al., in preparation) and so it was chosen as a host for anthocyanin biosynthesis.

While ANS was successfully expressed from the high-copy number vector using the NIsin Controlled Expression system NICE (Kuipers et al., 1998), high expression levels of 3GT proved more difficult in *L. lactis* cells. The overproducer strain showed increased cell aggregation and sedimentation presumably due to depletion of the intracellular UDP-Glu pool which is a substrate for 3GT, but also plays a crucial role in cell wall synthesis; strong expression of 3gt would reduce available UDP-Glu which may interfere with proper cell wall maintenance in *L. lactis*. To obtain reduced expression of 3gt, one copy was integrated in the chromosome of *L. lactis* Ldh-under control of the constitutive lactococcal promoter P*usp45*. Activity of the enzyme was verified by an *in vitro* enzymatic assay. The combination of the native constitutive promoter from *L. lactis* with a nisin-inducible system allowed us to engineer an anthocyanin production strain with high ANS activity, and a moderate 3GT expression which resolved growth difficulties related to UDP-Glu pool reduction. The resulting strain *L. lactis* NZLdh$^-$*3gt*$_{opt}$ pANS$_{opt}$ (NZAnt) was then tested for the ability to synthesize anthocyanins (Fig. 1).

3.2. Pigment production by L. lactis NZAnt: cell wall permeabilization strategies

When the two functional enzymes were expressed together in *L. lactis* cells and the substrate was added, a red colouration of the medium could be detected after 16 h of incubation. Gram-positive bacteria possess a thick cell wall and this polysaccharide-rich layer tends to retain polyphenols (Fernandes et al., 2014; de Freitas et al., 2017). To increase product yield *in vivo* and to facilitate diffusion of the substrate and products through lactococcal cell envelope, multiple cell wall permeabilization strategies were tested. Potential permeabilizing compounds were added to the production medium. Since peptidoglycan comprises a very thick layer in the cell wall of Gram-positive bacteria, lysozyme was tested as this enzyme cleaves the glycosidic bond between *N*-acetyl-muramic acid (NAM) and *N*-acetyl-glucosamine (NAG) in the peptidoglycan. Besides lysozyme, various permeabilizing compounds such as detergents Tween-80, Tween-20 and Triton X-100 were tested for effectiveness. Also, the antibacterial peptide nisin S was tested due to its ability to make pores in cytoplasmic membranes. Finally, we tested the influence of ethanol and butanol at a final concentration of 6% in the medium, since these compounds also affect the structure of the membrane (Fig. 2).

Assessment of the red colour intensity of the cultures can be used as a direct indicator of the amount of anthocyanins produced as a good correlation has been observed between the colour intensity and the actual anthocyanin concentration (Lim et al., 2015). To quantify the pigment production, cells were collected by centrifugation and disrupted by bead beating in acidified methanol. The absorption intensity of these red extracts was then analysed using an UV–Vis spectrophotometer at wavelength 515 nm (Supplementary Fig. 2). An addition of lysozyme and nisin improved the accumulation of red pigments in the cultures presumably due to enhanced diffusion of the substrate into the cell. These pigments were identified as anthocyanins by HPLC analyses (see Section 3.4). Thus, addition of 2.5 ng/ml of nisin in combination with 1 mg/ml of lysozyme to the production medium was chosen as the standard anthocyanin production conditions. Addition of relatively high concentration of lysozyme was required to compensate for its lower enzymatic activity in acidic production medium (pH 5) (Thammasirirak et al., 2007). Microscopy and cell plating experiments showed that even in the presence of cell wall degrading/distorting compounds for 64 h, *L. lactis* cells retained their shape and remained viable (Supplementary Fig. 3).

3.3. Production of anthocyanins in L. lactis pellicle-negative strain

It has been shown that *L. lactis* cells are covered with a polysaccharide layer which is covalently linked to the peptidoglycan (Chapot-Chartier et al., 2010). This pellicle consists of repeating hexasaccharide -NAG-rhamnose-NAG-glucose-galactose-glucose-phosphate- units linked by phosphodiester bonds. Strains that do not synthesize the wild-type pellicle are resistant to phage sk1 since this polysaccharide polymer was shown to serve as a receptor for phage adsorption. Phage sk1 - resistant pellicle mutants appear when the

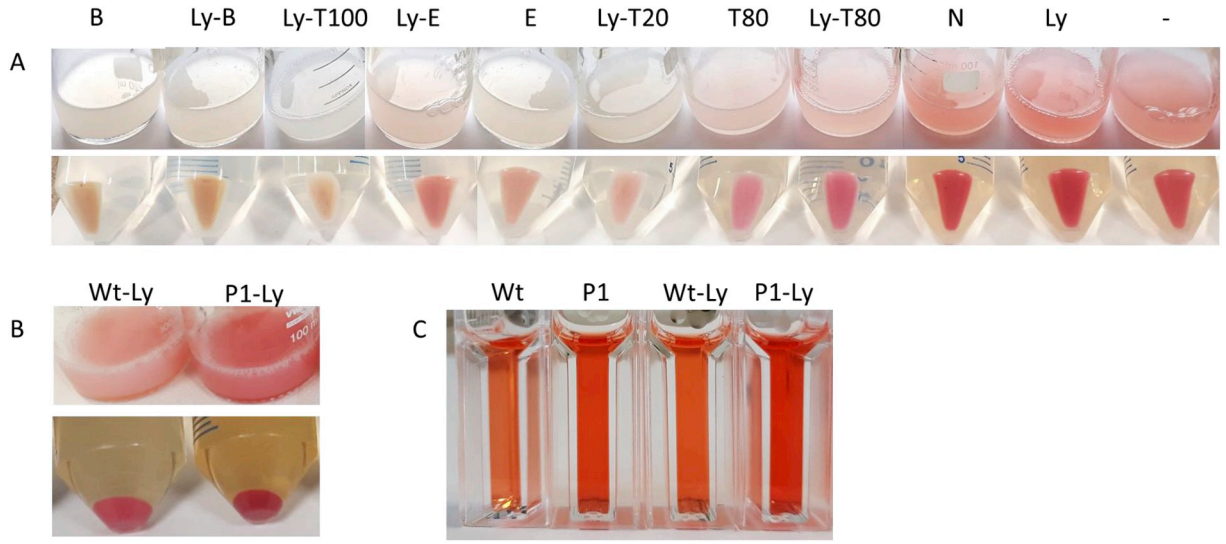

Fig. 2. Effect of various permeabilizing treatments on production of pigments in lactococcal cell cultures. (A) Pigment production in *L. lactis* NZpANS in the presence of one or a combination of cell wall permeabilization agents: B, butanol, E, ethanol; Ly, lysozyme; T20, Tween-20; T100, Triton X-100; N, nisin; T80, Tween-80. Production cultures (20 ml) consisting of production medium and *L. lactis* cells (above) and cell pellets with the production medium after centrifugation (below) are shown. (B) Disruption of pellicle improves pigment production in *L. lactis* NZpANS cells. wt, *L. lactis* NZpANS; P1, *L. lactis* NZpANS derivative carrying an IS element in the pellicle synthesis cluster, -Ly, strains incubated in production medium with lysozyme. Note that more pigment accumulates in the production medium of the pellicle mutant. (C) Crude extracts of the strains in cuvettes.

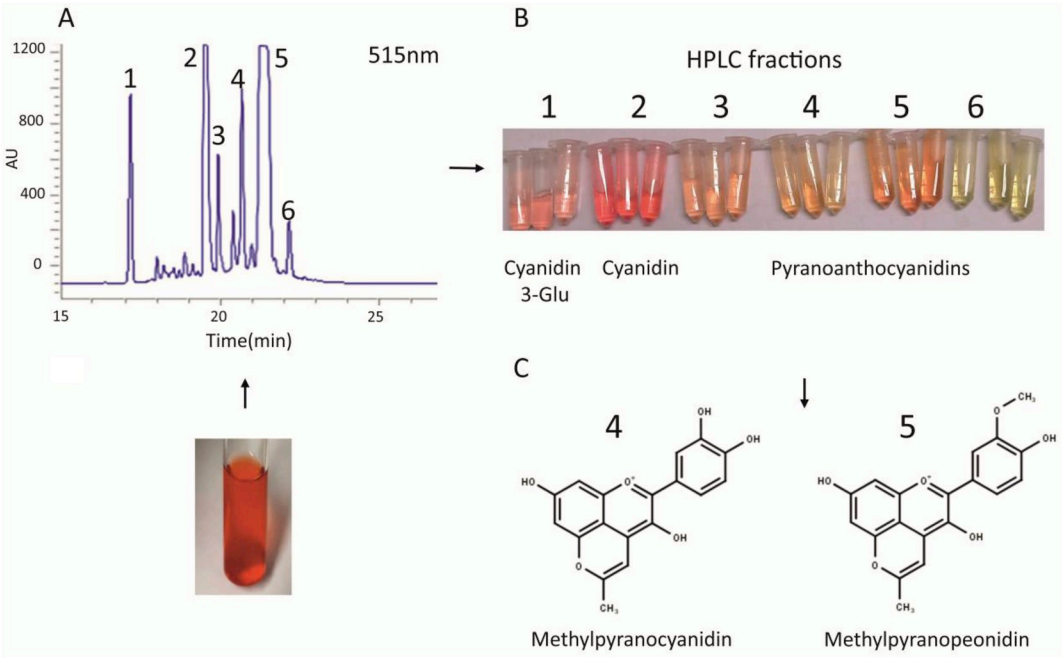

Fig. 3. Production of pigments in the engineered *L. lactis* strain NZAnt. (A) HPLC chromatogram of methanolic crude extracts of *L. lactis* NZAnt after incubation with (+)-catechin overnight. (B) Separated fractions collected show different colours. (C) Besides the expected red compounds, cyanidin 3-*O*-glucoside (peak 1) and cyanidin (2), the engineered *L. lactis* strains produced orange pyranoanthocyanidins (peaks 4 and 5) and other unidentified pigments (peaks 3 and 6). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

pellicle synthesis cluster is disrupted (Chapot-Chartier et al., 2010).

We reasoned that inactivation of polysaccharide pellicle would facilitate diffusion of (+)-catechin into *L. lactis* cells without addition of lysozyme to the production medium. To that end pellicle-negative derivative was selected as sk1-resistant isolate and then transformed with an ANS-harbouring vector. Genetic analyses of the strain revealed that it had acquired IS*981* element integrated in the putative outer-surface protein-encoding gene *llmg_0026*. Llmg_0226 was proposed to perform an essential role in the synthesis of the polysaccharide pellicle in *L. lactis* (Chapot-Chartier et al., 2010). The phage-resistant NZpANS isolate was tested for its ability to accumulate pigments. In line with our hypothesis, the pellicle mutant performed better than the control strain even in the absence of lysozyme. However, addition of lysozyme increased intracellular and extracellular pigment yields in the pellicle-negative strain. (Fig. 2, Supplementary Fig. 4). Since the lactococcal pellicle is covalently linked to the peptidoglycan, the lack of this polysaccharide layer presumably affects cell wall organization, and consequently renders it more permeable for (+)-catechin, thus facilitating its diffusion into the cell.

Alternatively, it was postulated for plant cells that anthocyanins and

similar polyphenolic compounds react with polysaccharides in cell walls impeding their extraction. Non-covalent interactions such as hydrogen bonding and hydrophobic interactions between cell wall sugar molecules and phenols occur (Fernandes et al., 2014; de Freitas et al., 2017). It is very plausible that similar interactions retain (+)-catechin and a portion of produced anthocyanins in the lactococcal cell wall.

3.4. Analysis reveals the presence of unexpected compounds

Visual analysis of *L. lactis* NZAnt anthocyanin production cultures after 16 h of incubation suggested that pink-red pigments were synthesized. The majority of the pigments accumulated intracellularly or in the cell wall, with lower amounts in the culture medium. To extract these pigments from cells, they were collected by centrifugation and disrupted by bead beating or sonication in acidified methanol. Subsequently, the composition of the extracts was analysed by high performance liquid chromatography (HPLC), monitoring A_{515} and A_{530}. The retention times of standard compounds were compared to those of obtained peaks. The engineered L. *lactis* strain NZAnt contained the expected peaks characteristic of cyanidin and cyanidin 3-*O*-glucoside but additional peaks of less polar compounds were detected at 515 nm and 530 nm indicating the presence of different pigments (Fig. 3). These new peaks were collected using HPLC and pink, orange and yellow compounds were obtained (Fig. 3).

Further analysis of the extracts by LC-MS provided accurate mass, MS2 fragmentation and PDA data which suggested that these unknown compounds are related to pyranoanthocyanins. Compound 4 corresponded to 2-(3,4-Dihydroxyphenyl)-3,8-dihydroxy-5-methylpyrano [4,3,2-de]chromen-1-ium. Compound 5 was predicted to result from the *O*-methylation of this compound to 2-(3-Methoxy-4-hydroxyphenyl)-3,8-dihydroxy-5-methylpyrano[4,3,2-de]chromen-1-ium. Based on the proposed structures, these compounds were assigned to the class of methylpyranoanthocyanidins. Curiously, no glycosylated derivatives of these pyroanthocyanidins were present in the crude extracts, suggesting that 3GT was not able to accept them as substrates.

In order to confirm these structures, 1D ^{1}H-NMR analysis was performed on purified compounds 4 and 5. The exact pyranoanthocyanidin structure determination by ^{1}H-NMR analysis was obscured by the mixture of pyrano- and furano-forms present in each sample and by the low quantity of purified material. Indeed, the amount of the presumed furano-forms increased when the samples were repeatedly freeze-dried and dissolved in acidic solvents during their preparation. Furanoanthocyanidins were also detected in a mixture with pyranoanthocyanidins by Lu et al. after acid-catalysed hydrolysis of pyranoanthocyanins (Lu and Foo, 2001, 2002). However, Compound 5 clearly differed from 4 by a distinct methoxy group peak, although the exact location of this moiety was not determined as the quantity of the material was insufficient to conduct 2D NMR experiments.

3.5. Synthesis of pyranoanthocyanidins from anthocyanidins does not require activity of heterologously expressed enzymes

Orange-yellow methyl-pyranoanthocyanins were first detected in the acetone extract of black currant seed residue. Their formation was proposed to arise from the reaction of black currant cyanidin- and delphinidin-glucosides with acetone (used as the extraction solvent) during the extraction process (Lu and Foo, 2001, 2002). Methylpyranoanthocyanins derived from malvidin-glucoside and -coumaroylglucoside were proposed to result from the reaction between anthocyanins and acetoacetic acid produced by yeasts during wine fermentation. Incubation for 7 days in a wine-like model solution (pH 3.2) of malvidin 3-coumaroylglucoside and acetoacetic acid yielded yellow methylpyranoanthocyanin (He et al., 2006). To test whether spontaneous reaction between cyanidin and lactococcal metabolite(s) was responsible for the appearance of pyranoanthocyanidins, *L. lactis* strains *L. lactis* NZAnt, NZpANS (expressing only ANS) and NZpNZ (empty vector) were grown as before. Nisin was added to all cultures for the induction of ANS expression (or as a control). The anthocyanidins cyanidin or pelargonidin were added to the medium instead of (+)-catechin. After an overnight incubation cells were harvested and their methanol extracts were analysed by HPLC.

Even in the absence of expressed plant enzymes, HPLC analyses of lactococcal cultures showed the presence of peaks of coloured derivatives with higher retention times suggesting the presence of pyranoanthocyanidins. When the cells expressed 3GT (NZAnt), a portion of cyanidin and pelargonidin was glycosylated, and peaks of cyanidin 3-*O*-glucoside or pelargonidin 3-*O*-glucoside, respectively, appeared in the chromatogram. Extracts from cells expressing only ANS contained relatively more pyranoanthocyanidins as there was no competition for the substrate with 3GT. Only the pyranoanthocyanidins-specifying peaks appeared in the extracts of the negative control NZLdh^{-} pNZ. Exclusion of 2OG or ascorbate from the production medium did not affect the formation of pyranoanthocyanidins. The results obtained by these experiments suggest that lactococcal metabolite(s) interact with anthocyanidins to form novel compounds – pyranoanthocyanidins - in accordance with the previous observations in wine.

3.6. Green tea as a substrate for the production of anthocyanins in L. lactis

The presence of various flavan-3-ols, such as (+)-catechin, can be observed in the composition of many plant-derived food products, such as cocoa, chocolate, nuts, fruits, vegetables and tea. To assess the feasibility of utilizing natural (+)-catechin sources as substrate for the production of anthocyanins by engineered *L. lactis* strains, two commercial green tea infusions (Lipton and Tetley Tea) were prepared. Then, using solid phase extraction (SPE) column STRATA C18, organic acids and minerals were removed and the eluting fractions were concentrated and enriched in total phenolic content. Besides (+)-catechin, green tea is rich in (+)-gallocatechin, (−)-epicatechin, (−)-epicatechin 3-*O*-gallate, (−)-epigallocatechin, (−)-epigallocatehin 3-*O*-gallate, procyanidins, kaempferol- and quercetin-glycosides. High concentrations of phenolic compounds are known to inhibit growth of bacteria. To determine whether tea (+)-catechin present in a mixture of various phenolic compounds could be accepted as a substrate by ANS expressed in *L. lactis*, freeze-dried tea preparations were dissolved in ethanol and added to the production medium instead of pure (+)-catechin. Anthocyanin production experiments were performed as described previously.

After an overnight incubation of *L. lactis* NZAnt and NZpANS with tea fractions, the production cultures accumulated a pink colour. LC-MS analysis of methanolic crude extracts showed that a range of coloured compounds was present (Fig. 4, Supplementary Fig. 1). Both types of tea preparations were converted into similar pigments by the engineered *L. lactis* strains. Cyanidin 3-*O*-glucoside, cyanidin and both previously found methylpyranoanthocyanidins (Fig. 3, compounds 4 and 5) were identified. The anthocyanidin delphinidin was formed presumably by ANS from (+)-gallocatechin and, to the best of our knowledge, this reaction has not yet been described for ANS. Four types of methylpyranoanthocyanidins were found: methylpyranocyanidin, methylpyranodelphinidin and their derivatives methylated on the B ring, methylpyranopeonidin and methylpyranopetunidin, respectively (Fig. 4, Supplementary Table 3). Of the pyranoanthocyanins, the methylated versions were the most abundant in the samples, suggesting very efficient methylation of their precursors and/or the higher stability of the methylated forms.

4. Discussion

Because of their biological potency and high abundance in plant foods, anthocyanins have been the focus of significant research interest during the last years. Their potential application in food or consumer products is, however, limited by their lack of stability.

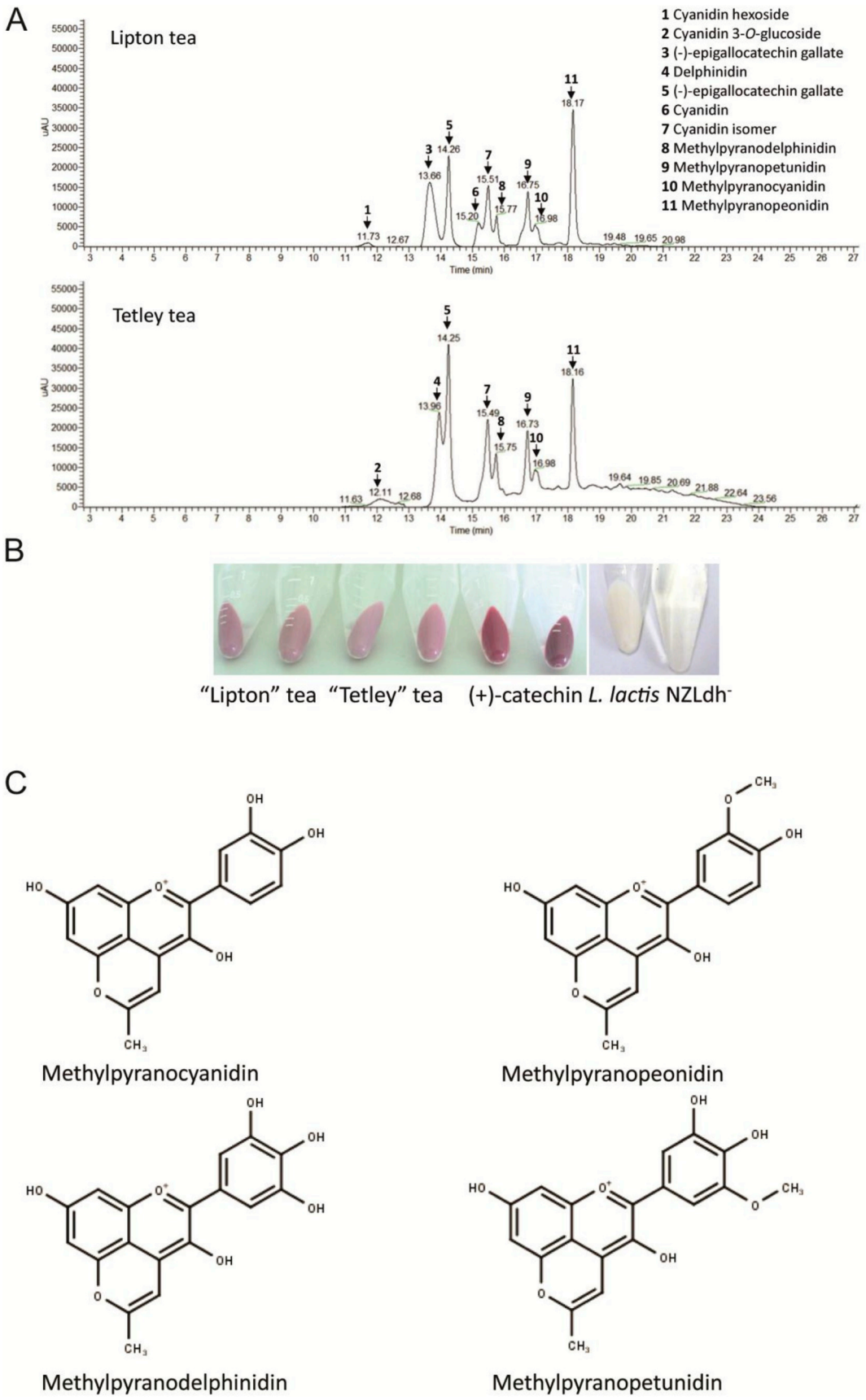

Fig. 4. Tea conversion by *L. lactis* NZAnt. (A) HPLC chromatograms at 510–530 nm absorbance of crude extracts derived from *L. lactis* NZAnt incubated with two commercial green tea products: Lipton and Tetley. (B) Anthocyanin production in *L. lactis* NZAnt cell pellets in the presence of various substrates. Control culture incubated with tea remains white-yellow. (C) Proposed structures of the pyranoanthocyanidins formed by NZAnt from green tea. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Pyranoanthocyanins are anthocyanin derivatives with much greater stability in high pH solutions conferred by the additional ring D which is formed between the OH group at C-5 and the C-4 of the anthocyanin pyran ring (Oliveira et al., 2011, 2014; de Freitas and Mateus, 2011; Morata et al., 2003). Chemical reaction between anthocyanins with small molecules released during wine fermentation were shown or proposed to be responsible for the chemical transformations of the former. These reactions involve condensation of anthocyanins or reactions of anthocyanins with pyruvic acid, vinylphenol, cinnamic acid derivatives, glyoxylic acid, acetaldehyde, acetone and acetoacetic acid. These events yield new pigment families that often stabilize wine colour and change it from the red-purple of young wines to the more orange-red of aged wines (He et al., 2006). Pyranoanthocyanins have been extensively studied under their natural synthesis conditions; e.g. grape-must fermentation with addition of various yeast or bacterial strains and other components (e.g., (+)-catechin, procyanidins) and analysis for the presence of pyranoanthocyanins and other pigments after days or even months of fermentation (Escott et al., 2016; Wang et al., 2018).

Pyranoanthocyanins are particularly interesting because of their contribution to the colour and the taste of red ageing wines, their antioxidant capacity and proposed health benefits. Since their discovery, various synthetic approaches have been developed to prepare pyranoflavylium cation analogues of the naturally occurring pyranoanthocyanins with potential application of these analogues as a synthetic cationic colourants with tunable colours (da Silva et al., 2018).

Here, we have shown that the engineered lactic acid bacterium *L. lactis* can quickly (in 16 h) transform green tea infusion into a range of potentially valuable anthocyanin derivatives. Two types of anthocyanins and four types of methylpyranoanthocyanidins were discovered in the cultures of *L. lactis* NZAnt. The low permeability of the lactococcal cell wall could potentially play a role in pyranoanthocyanidin formation as newly formed anthocyanidins are kept inside the cell where they react with the intermediates of cellular metabolism to form new pigments. Although similar interactions take place during winemaking and maturation, they take much longer (e.g. days or even months).

Using a Gram-positive anaerobic bacterium *L. lactis* for anthocyanin biosynthesis appears to have distinct features in comparison to using the well-established Gram-negative host *E. coli*. Lactococci proved to be robust at low pH and in high flavonoid concentrations, remaining viable throughout quite harsh production conditions. Although the total pigment yield (around 1.5 mg/L with approx. Half of it specifying cyanidin) was not as high as that of engineered *E. coli* strains (the highest being reported 350 mg/L) (Lim et al., 2015) we have investigated multiple strategies to potentially improve it. Besides codon optimization and promoter tuning, cell wall engineering can be employed to increase the pigment production. Intracellular polyphenols can be released to the medium by inducing the lysis of lactococcal cells at the end of the production phase using an inducible lysin-holin system (de Ruyter et al., 1997). Importantly, in contrast to the reported *E. coli* strains, *L. lactis* was able to produce a range of pigments from a raw preparation of a relatively cheap and abundant substrate, green tea, thus opening up the possibility to utilize other flavan-3-ol-rich sources as feedstock for the production of health-beneficial colorants.

High rates of anthocyanidin conversion into pyranoanthocyanidins might partly be responsible for the low amount of glycosylated anthocyanidins produced. Homeostasis of the cell wall building block UDP-glucose, which is also the substrate for 3GT seems to be very important for *L. lactis*, since increasing the copy number of 3gt negatively affected cell growth. Overexpression of UTP-glucose-1-phosphate uridylyltransferase (UDP-glucose pyrophosphorylase) encoding *galU* was previously shown to increase UDP-glucose and UDP-galactose concentration by eightfold (Boels et al., 2001). Our attempt to increase the UDP-glucose pool by *galU* overexpression did not have a significant effect on cyanidin glycosylation. Co-localization of the enzymes ANS and 3GT could be an alternative strategy to increase glycosylation of anthocyanidins. To co-localize and optimize the concentrations of enzymes and their substrates plant cells synthesize anthocyanins in multienzyme complexes called metabolons. Membrane lipid domains, – lipid rafts, - constitute platforms for the assembly of metabolons (Jorgensen et al., 2005; Crosby et al., 2011). Formation of similar synthetic metabolons on the bacterial cell membrane could provide many advantages; they would allow for the channelling of an intermediate that is formed at one active site of an enzyme to the active site of next enzyme decreasing the transit time, reduce dilution of intermediates and secure swift conversion of unstable compounds preventing their diffusion into the surrounding cell matrix.

Methylation of pyranoanthocyanidins, presumably by a lactococcal methyltransferase, to form methylpyranopeonidin and methylpyranopetunidin was an unexpected observation. Analysis of the *L. lactis* NZ9000 genome sequence revealed that no enzymes displayed significant homologies to anthocyanin *O*-methyltransferase which catalyses anthocyanin methylation in plants. Out of 42 putative or confirmed lactococcal methyltransferases only one possesses a distinct motif which allocates it to the family 3 of *O*-methyltransferases, and is designated LLNZ_09825, or YrrM. The family includes catechol *O*-methyltransferase and caffeoyl CoA-*O*-methyltransferase. Like anthocyanin *O*-methyltransferase they use *S*-adenosyl-L-methionine (SAM) as a substrate for methyl transfer. Catechol *O*-methyltransferase catalyses methylation of various endogenous and xenobiotic compounds including flavonoids in eukaryotes. The enzyme was shown to methylate molecules with a catechol moiety, such as catechin and procyanidins, preferentially in the *meta* (3-*O*-) position (Weinert et al., 2012). Methylation of the catecholic moiety reduces the antioxidant capacity of the molecule suggesting that the biological function of methylation might be related to deactivation of the potentially reactive center (Weinert et al., 2012). *L. lactis* has been postulated to originate from the plant niche before adaptation to life in milk. It is possible that the ability to methylate and metabolize pyranoanthocyanidins that display antibacterial activity is related to life on plants. The same hypothesis might hold true for the ability of this bacterium to withstand high concentrations of flavonoids.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2019.04.002.

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